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(54) Title: BIOSYNTHETIC CONSTRUCTS OF TGF- β			
(57) Abstract Disclosed are constructs of truncated transforming growth factor-beta (TGF- β) produced by expression of recombinant DNA in a prokaryotic host cell. These constructs include at least one polypeptide chain of fewer than about 112 amino acids and fewer than 9 cysteine residues. The sequence of amino acids in these constructs is sufficiently <i>duplicative of the sequence of native transforming growth factor-beta</i> such that it is capable of inducing an anti-proliferative effect on mammalian epithelial cells <i>in vitro</i> . Also disclosed are methods of producing analogs of TGF- β using recombinant DNA technology, and methods of using such analogs.			

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BIOSYNTHETIC CONSTRUCTS OF TGF- β Background of the Invention

This invention relates to biosynthetic peptide constructs of transforming growth factor-beta, to synthetic genes encoding polypeptides having transforming growth factor-beta-like biological activity, to methods of producing such synthetic genes using recombinant DNA technology, and to the use of such biosynthetic peptide constructs as regulators of cell proliferation and growth.

Transforming growth factor-beta (TGF- β) is a multifunctional peptide regulator of activity involving cellular or tissue response to injury or stress. This factor has the ability to stimulate cell proliferation in cells of mesenchymal origin, while also being able to inhibit the growth of epithelial cells, embryonic fibroblasts, endothelial cells, and T and B lymphocytes. In addition, TGF- β has a number of other regulatory activities which appear to be related uniquely to the specialized function of a particular cell type. For example, TGF- β stimulates the production of matrix components, e.g., inhibiting the synthesis and secretion of proteolytic enzymes which act on these components, thereby regulating the synthesis and degradation of extracellular matrix (for a review, see, e.g., Sporn et al. (1987) J. Cell Biol. 105:1039-1045). TGF- β -type activities have been identified in many normal fibronectin- and collagen-producing

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fibroblasts, as well as tissues such as kidney (Roberts et al. (1983) Biochem. 22:5692-5698), placenta (Frolik et al. (1983) Proc. Natl. Acad. Sci. (USA) 80:3676-3680), and platelets (Childs et al. (1982) Proc. Natl. Acad. Sci. (USA) 79:5312-5316; and Assoian et al. (1983) J. Biol. Chem. 258:7155-7160), as well as in tumor cells (see, e.g., Roberts et al. (1980) Proc. Natl. Acad. Sci. (USA) 77:3494-3498).

There are four known molecular configurations of TGF- β , each having an apparent molecular weight of about 25,000 daltons. Three of these species result from homodimeric (TGF- β 1, TGF- β 2) and heterodimeric (TGF- β 1.2) combinations of the monomeric subunits, β 1 and β 2. The fourth species is a homodimer of a β 3 subunit. Each subunit is processed from a precursor of about 390 amino acids, and the mature subunit protein includes approximately 112 amino acids of its carboxy terminus. The β 1 and β 2 subunits have about a 70% amino acid sequence homology in their N-terminal portions, and are highly conserved between species. The deduced amino acid sequence of TGF- β 3 shares about 80% homology with types β 1 and β 2, with many of the differences being conservative substitutions. TGF- β 1 was originally isolated from human platelets and placenta (EP 0128849), and bovine kidney (Roberts et al. ibid.). TGF- β 2 was originally identified as cartilage-inducing factors (CIF) isolated from bovine bone (US 4,774,228). TGF- β 1.2 has been found in porcine platelets and other cells which coexpress the β 1 and β 2 chains (Cheifetz et al. (1988) J. Biol. Chem. 263:10783-10789). TGF- β 3 has been identified

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in both human and chicken (Duke et al. (1985) Proc. Natl. Acad. Sci. (USA) 85:4715-4719).

The TGF- β 's belong to a larger gene family, the members of which encode structurally similar proteins that have similar regulatory activities (reviewed in Massague (1987) Cell 49:437-438). Included in this family are: (1) Vg1, a protein involved in mesoderm formation during Xenopus development; (2) decapentaplegic complex (DPP), a polypeptide encoded by a Drosophila gene responsible for development of the dorsoventral pattern in the embryo; (3) OPl, a region of a native osteogenic protein sequence encoded by exons of a genomic DNA sequence retrieved by applicants; (4) cartilage inducing factors (CIFs) isolated from bovine bone (US 4,774,228); (5) mammalian osteogenic bone matrix proteins CBMP-2a, CBMP-2b, and CBMP-3, discovered by applicants (see WO89/01453); and (6) β -inhibin-a and b, gonadal proteins that suppress pituitary secretion of follicle stimulating hormone. All of these proteins are believed to dimerize during refolding, and are inactive when reduced to the monomeric form. In addition, many include portions of a common precursor peptide.

Identification of the regulatory activities of the proteins in the TGF- β family, and the elucidation of their amino acid sequences, have resulted in research efforts directed to the production of these proteins by recombinant means. For example, EP 0200341 discloses nucleic acid sequences encoding native TGF- β and precursors thereof which can be expressed in a host eukaryotic

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cell transformed therewith. EP 0150572 discloses the manufacture of structural genes coding for TGF- β 1 and analogs thereof and the expression of these genes in microorganisms. However, the design and expression of consensus protein constructs having considerable sequence homology with a number of the proteins in the TGF- β family, and displaying TGF- β -like activity, has heretofore not been contemplated.

Accordingly, it is an object of this invention to provide novel analogs of TGF- β having TGF- β biological activities. Another object is to provide an efficient method of producing novel, active TGF- β analogs. Yet another object is to provide genes encoding novel, non-native, TGF- β species and methods for their production using recombinant DNA techniques. Another object is to provide novel truncated forms of TGF- β and structural designs for proteins with TGF- β biological activity. A further object is to provide methods of regulating cell proliferation using TGF- β analogs.

These and other objects and features of the invention will be apparent from the description, drawings, and claims which follow.

Summary of the Invention

It has been discovered that forms of native TGF- β which have been truncated at the N-terminus, and which have fewer than the native number of cysteine residues demonstrate TGF- β -like biological activity, including the ability to induce an anti-proliferative effect on mammalian epithelial cells in vitro. It has also been unexpectedly discovered that truncated analogs of other structurally similar proteins in the TGF- β family known to have unrelated biological activities also possess this TGF- β -like activity. These discoveries enabled the design and construction of DNAs encoding novel, non-native protein constructs which individually and combined are capable of inhibiting the proliferation of mammalian epithelial cells in culture.

Thus, in one aspect, the invention comprises a truncated TGF- β analog produced by expression of recombinant DNA in a host cell and capable of inducing an antiproliferative effect in mammalian epithelial cells in vitro. This protein construct includes two polypeptide chains, each including a biologically active domain, and each having fewer than 9, and preferably 6 or 8, cysteine (Cys) residues. It may further be characterized as being unglycosylated.

In another aspect, the invention comprises a protein produced by expression of recombinant DNA in a prokaryotic host cell and including a pair of polypeptide chains of fewer than about 112 amino

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acids each. The sequence of amino acids in each chain is sufficiently duplicative of the sequence of TGF- β such that the protein is capable of inducing an anti-proliferative effect on mammalian epithelial cells in vitro. Preferably, the polypeptide chain contains fewer than 9, and more preferably 6 or 8, cysteine residues, and further, may be unglycosylated.

The cysteine residues are involved in the formation of intra- and inter-chain disulfide bonds (folding), the correct formation of which results in an active construct having TGF- β -like activity. In eucaryotes, the synthesis and proper folding of the protein can occur at least within those cells known to express TGF- β ; in prokaryotic cells, folding must be performed in vitro, a difficult feat in that any number of combinations of disulfide linkages exist between two polypeptide chains, each having less than 9, and preferably 6 or 8 cysteine residues. An important aspect of this invention is the discovery that truncated constructs of the type described herein may be post-translationally modified and folded (by oxidation) in vitro to produce TGF- β -like activity.

Several forms of TGF- β monomers are known in nature, β 1, β 2, and β 3. Investigation of the properties and structure of these native forms enabled the development of a rational design for non-native (i.e., not known to be expressed in nature), truncated protein constructs which also are capable of differentially regulating cell proliferation in various cell types. Further, upon

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examination of a number of unrelated proteins with some amino acid sequence homology, it was unexpectedly discovered that they, too, possess TGF- β -like activity.

Based on this knowledge, a series of consensus DNA sequences were designed with the goal of producing active TGF- β analogs. The sequences were based on partial amino acid sequence data obtained from native TGF- β species and from observed homologies with genes reported in the literature encoding proteins of the TGF- β family (including Vg1 and DPP), or on the amino acid sequences they encode, having a presumed or demonstrated developmental function. Several of the biosynthetic consensus sequences have been expressed as fusion proteins in prokaryotes, purified, cleaved, refolded, applied to a mammalian in vitro assay system, and shown to have TGF- β -like anti-proliferative activity.

In preferred aspects of the invention, the proteins encoded by these consensus sequences include the generic amino acid sequences:

```
      10      20      30      40      50
CXXXLYXXFXXDXGWX $\theta$ WXXXPXGYAXXCXGXCPXXXX $\theta$ X $\theta$ XXX $\theta$ X $\theta$ XXX
      60      70      80      90     100
XXLXXXXXPXXX $\theta$ XXXCCVXXXXXXXXXXXXXXXXXXXX $\theta$ XXMXVXXCXCX
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and

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      10      20      30      40      50
LYXXFXXDXGWXØWXXXPXGYAXXCXGXCPXXXXØØXØXXXØØXØXXX
      60      70      80      90      100
XXLXXXXXPXXXØXXXCCVXXXXXXXXXXXXXXXXXXXXXXXXØXXMXVXXCXCX

```

wherein the letters indicate the amino acid residues of standard single letter code, each "X" independently represents one of the naturally occurring amino acid or a derivative thereof, and each "Ø" independently represents an amino acid or a peptide bond.

The currently preferred active peptide constructs comprise amino acid sequences derived from the three monomeric subunits of TGF-β (1, 2, and 3), DPP, and OP1. The amino acid sequences of these proteins are set forth in FIGURE 1 relative to the sequence of TGF-β1. Preferred amino acid sequences within the foregoing generic sequences are:

```

      10      20      30      40      50
CCVRQLYIDFRKDLGWK-WIHEPKGYHANFCLGPCPYIWS--L-DTQ--Y-SKV
  L P      KR      N      A A      S      H R
      Q      V      Y      S      R      A      T      T
KK H  VE  - V  QN  IA Q  M  Y Y E  PLTEI NGSN      AIL
RRHS      S      DD  V  L  D Y  H K  F  ADHF S      V

      60      70      80      90      100
LALYNQHNP GAS-AAPCCVPQALEPLIVYYVGRKPKVEQL-SNMIVRSCKCS
  S  TI  E      S      S D      T L  I  KT  I      K
  G      L
QT VHS E  D-IPL      TKMS ISM F DNNDNV LRHYE  A DE G R
      NN      K V KA      Q DSA      LNDQST      KN QE T VG

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and

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      10      20      30      40      50
LYIDFRKDLGWK-WIHEPKGYHANFCLGPCPYIWS--L-DTQ--Y-SKV
P      KR      N      A A      S      H R
      Q      V      Y      S      R      A      T      T
VE - V QN IA Q M Y Y E PLTEI NGSN AIL
      S      DD V L D Y H K F ADHF S      V

      60      70      80      90      100
LALYNQHNP GAS-AAPCCVPQALEPLPIVYYVGRKPKVEQL-SNMIVRSCCKCS
S TI E S S D T L I KT I K
G L V
QT VHS E D-IPL TKMS ISM F DNNDNV LRHYE A DE G R
      NN K V KA Q DSV LNDQST KN QE T VG

```

wherein, at each position where more than one amino acid is shown vertically, any one of the amino acids shown may be used alternatively in various combinations, and "-" and "--" represent a peptide bond. Note that the numbering of amino acids is selected solely for purposes of facilitating comparisons among alternative sequences. These generic sequences have fewer than 9, and preferably 6-8 cysteine residues where inter- and/or intramolecular disulfide bonds can form, and contain other critical amino acids which influence the tertiary structure of the proteins. Similar structural features are found in the above named known proteins of the TGF- β family whose amino acid sequences previously have been published. However, of these only the TGF- β species (1, 2, and 3) have been described as capable of inducing an anti-proliferative effect in mammalian epithelial cells in vitro.

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Particular useful sequences include analogs having the following amino acid sequences:

TGF- β 1

10 20 30 40 50
CCVRQLYIDFRKDLGWKWIHEPKGYHANFCLGPCPYIWSLDTQYSKVLAL
60 70 80 90 100
YNQHNPGASAAPCCVPQALEPLPIVYYVGRKPKVEQLSNMIVRSCKCS;

TGF- β 2

10 20 30 40 50
CCLRPLYIDFKRDLGWKWIHEPKGYNANFCAGACPYLWSSDTQHSRVLSLY
60 70 80 90 100
NTINPEASASPCCVSQDLEPLTILYYIGKTPKIEQLSNMIVKSKCS;

TGF- β 3

10 20 30 40 50
CCVRPLYIDFRQDLGWKWVHEPKGYANFCSGPCPYLRSADTTHTSTVLGL
60 70 80 90 100
YNTLNPEASASPCCVPQDLEPLTILYYVGRTPKVEQLSNMVVKSKCS;

V α 1

10 20 30 40 50
CKKRHLYVEFKDVGWQNWVIA PQGYMANCYGECPYPLTEILNGSNHAIL
60 70 80 90 100
QTLVHSIEPEDIPCCVPTKMSPVAMLYLNDQSTVVLKNYQEMTVVGCGR;

DPP

10 20 30 40 50
CRRHSLYVDFSDVGWDDWIVAPLGDAYYCHGKCPFPLADHFNSTNHAVV
60 70 80 90 100
QTLVNNNNPGKVPKACCVPTQLDSISMLFYDNNNDNVVLRHYENMAVDECGCR;

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and

10 20 30 40 50
CCVRQLYIDFKRDLGWKWWHEPKGYAANFCAGACPYLWSADTQHRSVLA
60 70 80 90
LYNTANPEASAAPCCVPQDLEPLTILYYVGRTPKVEQLSNMVVKSCCKCS.

or more truncated analogs such as:

TGF-B1

10 20 30 40 50
LYIDFRKDLGWKWIHEPKGYHANFCLGPCPYIWSLDTQYSKVLAL
60 70 80 90 100
YNQHNPGASAAPCCVPQALEPLPIVYYVGRKPKVEQLSNMIVRSCKCS;

TGF-B2

10 20 30 40 50
LYIDFRKDLGWKWIHEPKGYNANFCAGACPYLWSSDTQHRSVLSLY
60 70 80 90 100
NTINPEASASPCCVSQDLEPLTILYYIGKTPKIEQLSNMIVKSCCKCS;

TGF-B3

10 20 30 40 50
LYIDFRQDLGWKWWHEPKGYANFCSGPCPYLRSADTTHSTVLGL
60 70 80 90 100
YNTLNPEASASPCCVPQDLEPLTILYYVGRTPKVEQLSNMVVKSCCKCS;

Vg1

10 20 30 40 50
LYVEFKDVGWQNWVIAPOGYMANCYGECPYPLTEILNGSNHAIL
60 70 80 90 100
QTLVHSIEPEDIPCCVPTKMSPVAMLYLNDQSTVVLKNYQEMTVVGCGR;

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DPP

10 20 30 40 50
LYVDFSDVGWDDWIVAPLGYDAYYCHGKCPFLADHFNSTNHAVV
60 70 80 90 100
QTLVNNNNPGKVPKACCVPTQLDSISMLFYDNNNDNVVLRHYENMAVDECGCR;

and

10 20 30 40 50
LYIDFKRDLGWKVVHEPKGYAANFCAGACPYLWSADTQHSRVLALYN
60 70 80 90 100
TANPEASAAPCCVPQDLEPLTILYYVGRTPKVEQLSNMVKSCCKCS.

The name given to each of these sequences designates the natural source DNA sequence encoding the amino acid sequence which, as far as applicants are aware, exhibits the most homology with the recited TGF- β analog.

The invention further includes DNA sequences encoding these constructs and a prokaryotic host cell engineered to express these DNA sequences. In a preferred aspect of the invention, the prokaryotic host cell (e.g. *E. coli*) is transfected with a vector including the TGF- β -encoding DNA sequence. The transformed cell is cultured to express the protein which is then purified and activated by oxidation in vitro. The protein so treated has the ability to induce an anti-proliferative effect on cultured mammalian epithelial cells.

The biosynthetic constructs disclosed herein may be used to regulate cellular activities such as proliferation and growth. In this regard, these

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constructs have wide potential clinical applications, for example, by controlling the proliferation of various tumor cell lines, or by enhancing the growth rate of T and B lymphocytes in immunosuppressed (e.g. Acquired Immunodeficiency Syndrome (AIDS) patients. The constructs also may be used in cell cultures to modulate growth of various types of eucaryotic cells.

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Brief Description of the Drawing

The foregoing and other objects of this invention, the various features thereof, as well as the invention itself, may be more fully understood from the following description, when read together with the accompanying drawings, in which:

FIGURE 1 is a comparison of the amino acid sequence of various proteins in the TGF- β family to TGF- β 1; and

FIGURES 2A and 2B are representations of a DNA sequence and corresponding amino acid sequence of a modified trp-LE leader sequence, two FB domains of protein A, an Asp-Pro cleavage site, and (A) the 6 Cys TGF- β 1 sequence, and (B) the 8 Cys TGF- β 1 sequence.

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Description

Nucleic acid sequences encoding truncated TGF- β analogs were designed based on sequence data reported in the literature, codons inferred from known amino acid sequences, and observations of partial homology with known genes of the TGF- β family. These sequences have been refined by comparison with the sequences present in certain regulatory genes from the TGF- β family.

The naturally occurring proteins of the TGF- β family are made as precursors, with a large and poorly conserved N-terminal domain and a characteristic C-terminal domain in which a pattern of 7 cysteines (Cys) residues is highly conserved. In addition to these Cys residues, certain other amino acids are found in members of the family very nearly in the same relative positions in sequence as set forth below:

```

      10      20      30      40      50
CXXXLXXFXDXGWXθWXXXPXGYAXXCXGXCPXXXθXθXXXθθXXX
      60      70      80      90     100
XXLXXXXPXXXθXXXCCVXXXXXXXXXXXXXXXXXXXXθXXMXVXXCXCX

```

wherein each X independently represents a naturally occurring amino acid, and θ or θθ represents an amino acid or peptide bond.

The N-terminal sequence of mature TGF- β and other related proteins contains a variable number of Cys residues which appear to be crosslinked among each other or with a residue of another amino acid chain, but not to Cys residues in the C-terminal

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domain. Maturation of the precursor to the mature form of these proteins occurs by trypsin-like cleavages between the precursor and the mature protein, and possibly also within the precursor form as other similar cleavage sites are present therein.

All members of the Vgl-related subgroup of the TGF- β family (including Vgl, DPP, OP1, CBMP-2a, CBMP-2b, and CBMP-3) share the feature of two basic residues (i.e., Lys-Lys, Arg-Arg, Arg-Lys) following the first Cys in the conserved C-terminal domain. The conserved double basic residues may represent another secondary maturation site. Cleavage by trypsin or related protease releases a C-terminal domain containing only 6 Cys residues. Since the precursor region of TGF- β contains up to 5 Cys residues which are not crosslinked to the C-terminal domain, the first of the 7 Cys residues may not be crosslinked to the C-terminal domain either. Therefore 6 Cys residues appear to be sufficient for a properly folded C-terminal domain.

In view of this disclosure, skilled genetic engineers can design and synthesize genes which encode a number of appropriate amino acid sequences. These genes can be expressed in various types of eucaryotic cells but, for reasons of efficiency are preferably produced in prokaryotic host cells, thereby providing large quantities of active synthetic proteins such as truncated analogs, muteins, fusion proteins, and other constructs, all mimicking the biological activity of native TGF- β , including the ability to induce an anti-proliferative effect on cultured mammalian epithelial cells.

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More specifically, the DNA sequences designed according to the above criteria and logic were constructed using known techniques involving assembly of oligonucleotides manufactured in a DNA synthesizer. The sequences may be expressed using well established recombinant DNA technologies in various prokaryotic host cells, and the expressed proteins may be cleaved from precursors, oxidized, and refolded in vitro for biological activity. This approach has been successful in producing a number of novel protein constructs not found in nature (as far as applicants are aware) which have TGF- β -like activity, i.e., the ability to induce an anti-proliferative effect on cultured mammalian epidermal cells.

The design and production of such biosynthetic proteins, and other material aspects concerning the nature, utility, how to make, and how to use the subject matter claimed herein will be further understood from the following non-limiting examples, which constitute the best method currently known for practicing the various aspects of the invention.

EXAMPLES

1. Consensus Sequence Design

Published amino acid sequences for TGF- β 2, TGF- β 3, Vg1, and DPP-C were used to determine which amino acids showed strong homology with the TGF- β 1 sequence. FIGURE 1 compares the amino acid sequences

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of these proteins with the sequence of TGF- β 1 (* denotes a match), and TABLE 1 summaries the extent of homology.

TABLE 1

<u>comparison</u>	<u>no. of matches</u>	<u>% homology</u>
TGF- β 2/TGF- β 1	78/115	67.8
TGF- β 3/TGF- β 1	85/115	73.9
DPP/TGF- β 1	34/115	29.6
Vg1/TGF- β 1	35/115	29.6

In determining an appropriate consensus amino acid sequence for TGF- β analogs, from which encoding nucleic acid sequences can be determined, the following points were considered: (1) the known amino acid sequence of natural source TGF- β 1, 2, and 3 are ranked highest; (2) where an amino acid in the sequence matches for all three proteins, it is used in the synthetic gene sequence; (3) matching amino acids in DPP and Vg1 are used; (4) if Vg1 or DPP diverge, but either one were matched by TGF- β 1, 2, or 3, this matched amino acid was chosen; and (5) where all sequences diverge, the amino acid residue alanine was chosen, provided that the secondary structure is maintained.

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Using these criteria, the preferred sequence is:

```
      10      20      30      40      50
CCVRQLYIDFKRDLGKWKVHEPKGYAANFCAGACPYLWSADTQHSRVLA
      60      70      80      90
LYNTANPEASAAPCCVPQDLEPLTILYYVGRTPKVEQLSNMVKSKCS.
```

In addition, the first consensus sequence was designed to preserve 8 of the disulfide crosslinks and the apparent structural homology among the related proteins, while the second more highly truncated consensus sequence was designed to preserve 6 disulfide bonds. That sequence is:

```
      10      20      30      40      50
LYIDFKRDLGKWKVHEPKGYAANFCAGACPYLWSADTQHSRVLALYN
      60      70      80      90     100
TANPEASAAPCCVPQDLEPLTILYYVGRTPKVEQLSNMVKSKCS.
```

2. Gene Preparation and Expression

The synthetic genes designed using the criteria set forth above are produced by assembly of chemically synthesized oligonucleotides. 15-100mer oligonucleotides are synthesized on a Biosearch DNA Model 8600 Synthesizer, and are purified by polyacrylamide gel electrophoresis (PAGE) in Tris-Borate-EDTA buffer (TBE). The DNA is then electroeluted from the gel. Overlapping oligomers are phosphorylated by T4 polynucleotide kinase, and then ligated into larger blocks which may also be

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purified by PAGE. Alternatively, natural gene sequences and cDNAs may be used for expression. The two resulting genes are shown as the latter portion of the fusion sequences in FIGURES 2A and 2B. The sequence shown in 2A is a truncated form of 2B; five amino acids at the N-terminus have been eliminated.

To enable the expression of the synthetic gene shown in FIGURE 2A in an E. coli host, the gene is modified by cassette mutagenesis. The N-terminus is replaced up to the ClaI site with a hinge region that provides for release of the TGF- β protein from the leader, preceded by a BamHI site for attachment to leader peptides. The modified gene is then attached to an FB-dimer leader at the BamHI site. The complete fusion gene is shown in FIGURE 2A.

The fusion gene is then inserted as an EcoRI to PstI fragment into an expression vector based on pBR322 and containing a synthetic tryptophan (trp) promoter/operator and a modified trp-LE (MLE) leader (which is similar to the one described by Huston et al. in Proc. Natl. Acad. Sci. (USA) (1988) 85:5879-5883, but having only a single EcoRI site at the hinge of the MLE leader). The vector is opened at the EcoRI and PstI restriction sites, and a FB-FB/TGF- β gene fragment is then inserted therebetween, where FB is fragment B of Staphylococcal Protein A. The resulting expression vector includes the TGF- β gene to a fragment encoding FB.

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3. Production of Active Analogs

The protein constructs are expressed in E. coli host strain JM101 (e.g.) grown in minimal medium (M9) after starvation for trp and induction by indoacrylic acid (IAA). The cells are lysed and the inclusion bodies collected by differential centrifugation. The fusion proteins are purified from the inclusion bodies by urea or guanidine solubilization. The FB sequence is then chemically cleaved from the TGF- β protein construct at the hinge region of the fusion protein. The hinge region has the sequence Asp-Pro-Asn-Gly which can be cleaved at the Asp-Pro site with dilute acid, or at the Asn-Gly site with hydroxylamine. The resulting cleavage products are passed through a Sephadryl-200HR column which separates most of the uncleaved fusion products from the TGF- β analogs.

Protein refolding is performed under the conditions of 50 mM Tris-HCl, pH 8.0, 3 M guanidine hydrochloride (GuHCl), 10 mM dithiothreitol (DTT), and 1-10 mM oxidized glutathione.

4. TGF- β Activity Assay

This assay is based on the ability of TGF- β to inhibit DNA synthesis in the mink lung epithelial cell line, ATCC no. CCL 64. A confluent culture of CCL-64 maintained in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 200 units/ml penicillin, and 200 μ g/ml streptomycin, is used to seed a 48-well cell culture plate at a cell density of 200,000 cells per well.

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When the culture becomes confluent, the media is replaced with 0.5 ml of EMEM containing 1% FBS and penicillin/streptomycin. The culture is incubated for 24 hours at 37°C. The TGF- β test samples in EMEM containing 0.5% FBS are then added to the wells, and the cells are then incubated for another 18 hours. After incubation, 1.0 μ Ci of 3 H-thymidine in 10 μ l is added to each well. The cells are incubated for 4 hours at 37°C. The media is then removed and the cells washed once with ice-cold phosphate-buffer saline. The DNA is precipitated by adding 0.5 ml of 10% TCA to each well and incubating at room temperature for 15 min. The cells are then washed three times with ice-cold distilled water and lysed with 0.5 ml 0.4 M NaOH. The lysate from each well is then transferred to a scintillation vial and the radioactivity recorded using a scintillation counter (Smith-Kline Beckman).

Each test sample is assayed in triplicate. A TGF- β control is included in each assay. The inhibition activity of each sample is expressed as the 50% effective dose (ED50), which is defined as the amount of material in ng/ml required to induce 50% reduction in maximal incorporation of 3 H-thymidine.

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The present embodiments are therefore to be considered in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing

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description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

What is claimed is:

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1. A truncated transforming growth factor-beta construct produced by expression of recombinant DNA in a host cell, capable of inducing an anti-proliferative effect in mammalian epithelial cells in vitro, and comprising two polypeptide chains, each of said chains comprising an active domain having fewer than 9 cysteine residues.
2. The construct of claim 1 wherein each of said active domains comprises 8 cysteine residues.
3. A protein produced by expression of recombinant DNA in a prokaryotic host cell, said protein comprising a pair of polypeptide chains, each of which has fewer than about 112 amino acids in a sequence sufficiently duplicative of the sequence of transforming growth factor-beta such that said protein is capable of inducing an anti-proliferative effect on mammalian epithelial cells in vitro.
4. The protein of claim 3 wherein each of said polypeptide chains comprises less than 7 cysteine residues.
5. The protein of claim 4 wherein each of said polypeptide chains comprises 6 cysteine residues.
6. The protein of claim 1 or 3 further characterized by being unglycosylated.

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7. The protein of claim 1 or 3 comprising the amino acid sequence:

```
      10      20      30      40      50
CXXXLYXXFXXDXGWXθWXXXPXGYAXXCXGXCPXXXXθθXθXXXθθXθXX
      60      70      80      90     100
XXLXXXXXPXXXθXXXCCVXXXXXXXXXXXXXXXXXXXXXXXXθXXMXVXXCXCX
```

wherein each "X" independently represents one of the naturally occurring amino acid or a derivative thereof, and each "θ" independently represents an amino acid or a peptide bond.

8. The protein of claim 1 or 3 comprising the amino acid sequence:

```
      10      20      30      40      50
LYXXFXXDXGWXθWXXXPXGYAXXCXGXCPXXXXθθXθXXXθθXθXX
      60      70      80      90     100
XXLXXXXXPXXXθXXXCCVXXXXXXXXXXXXXXXXXXXXXXXXθXXMXVXXCXCX
```

wherein each "X" independently represents one of the naturally occurring amino acid or a derivative thereof, and each "θ" independently represents an amino acid or a peptide bond.

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9. The protein of claim 7 comprising the amino acid sequences:

```

      10      20      30      40      50
CCVRQLYIDFRKDLGWK-WIHEPKGYHANFCLGPCPYIWS--L-DTQ--Y-SKV
  L P      KR      N      A A      S      H R
      Q      V      Y      S      R A T
KK H VE - V QN IA Q M Y Y E PLTEI NGSN AIL
RRHS      S      DD V L D Y H K F ADHF S      V

      60      70      80      90      100
LALYNQHNPNGAS-AAPCCVPQALEPLPIVYYVGRKPKVEQL-SNMIVRSCKCS
  S TI E S S D T L I KT I      K
  G L      V
QT VHS E D-IPL TKMS ISM F DNNDNV LRHYE A DE G R
      NN K V KA Q DSA LNDQST KN QE T VG

```

wherein, at each position where more than one amino acid is shown, any one of said amino acids shown may be in that position, and "-" and "--" represent a peptide bond.

10. The protein of claim 9 comprising the amino acid sequence:

```

      10      20      30      40      50
CCVRQLYIDFRKDLGWKWIHEPKGYHANFCLGPCPYIWSLDTQYSKVLAL
      60      70      80      90      100
YNQHNPNGASAAPCCVPQALEPLPIVYYVGRKPKVEQLSNMIVRSCKCS.

```

11. The protein of claim 9 comprising the amino acid sequence:

```

      10      20      30      40      50
CCLRPLYIDFKRDLGWKWIHEPKGYNANFCAGACPYLWSSDTQHRSRVLSLY
      60      70      80      90      100
NTINPEASASPCCVSQDLEPLTILYYIGKTPKIEQLSNMIVKSKCS.

```

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12. The protein of claim 9 comprising the amino acid sequence:

```
      10      20      30      40      50
CCVRPLYIDFRQDLGWKWWHEPKGYIANFCSGPCPYLRSADTTTHSTVLGL
      60      70      80      90     100
YNTLNPEASASPCCVPQDLEPLTILYYVGRTPKVEQLSNMVVKSCCKS.
```

13. The protein of claim 9 comprising the amino acid sequence:

```
      10      20      30      40      50
CKKRHLVVEFKDVGWQNWVIAPOGYMANCYGECPYPLTEILNGSNHAILQ
      60      70      80      90     100
TLVHSIEPEDIPLPCCVPTKMSPVAMLYLNDQSTVVLKKNYQEMTVVGCGCR.
```

14. The protein of claim 9 comprising the amino acid sequence:

```
      10      20      30      40      50
CRRHSLYVDFSDVGWDDWIVAPLGDAYYCHGKCPFPLADHFNSTNHAVVQ
      60      70      80      90     100
TLVNNNNPGKVPKACCVPTQLDSISMLFYDNNNDNVVLRHYENMAVDECGCR.
```

15. The protein of claim 9 comprising the amino acid sequence:

```
      10      20      30      40      50
CCVRQLYIDFKRDLGWKWWHEPKGYAANFCAGACPYLWSADTQHSRVLALYN
      60      70      80      90     100
TANPEASAAPCCVPQDLEPLTILYYVGRTPKVEQLSNMVVKSCCKS.
```

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16. The protein of claim 8 comprising the amino acid sequences:

```

      10      20      30      40      50
LYIDFRKDLGWK-WIHEPKGYHANFCLGPCPYIWS--L-DTQ--Y-SKV
P      KR      N      A A      S      H R
      Q      V      Y      S      R      A      T      T
VE - V QN IA Q M Y Y E PLTEI NGSN AIL
S      DD V L D Y H K F ADHF S V
FF S      I N      S      E      SHI GT G LSF ST I
      -      T      G      S      AY VP AS T
      E AR      A      NSYMNAT
      P      F      L
      Q      D
K A      SE S SF      Q MPKS KP

      60      70      80      90      100
LALYNQHNP GAS-AAPCCVPQALEPLPIVYYVGRKPKVEQL-SNMIVRSCKCS
S      TI E S S D T L I KT I K
G      L      V
QT VHS E D-IPL TKMS ISM F DNNDNV LRHYE A DE G R
NN K V KA Q DSV A LNDQST KN QE T VG
NHYMRGHSPFANL S R M GQ I K DI E
Q LN GTKVN I T F EY VP A
F T A NA V S K R A H
V - E E EK D
S Y
I RA GVVP G E E V A

```

wherein, at each position where more than one amino acid is shown, any one of said amino acids shown may be in that position, and "-" and "--" represent a peptide bond.

17. The protein of claim 16 comprising the amino acid sequence:

```

      10      20      30      40      50
LYIDFRKDLGWKWIHEPKGYHANFCLGPCPYIWSLDTQYSKVLAL

      60      70      80      90      100
YNQHNP GASAAAPCCVPQALEPLPIVYYVGRKPKVEQLSNMIVRSCKCS.

```

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18. The protein of claim 16 comprising the amino acid sequence:

```
      10      20      30      40      50
LYIDFKRDLGWKWIHEPKGYNANFCAGACPYLWSSDTQHSRVLSLY
      60      70      80      90     100
NTINPEASASPCCVSQDLEPLTILYYIGKTPKIEQLSNMIVKSKCS.
```

19. The protein of claim 16 comprising the amino acid sequence:

```
      10      20      30      40      50
LYIDFRQDLGWKQVHEPKGYANFCGPGPYLRSADTTHSTVLGL
      60      70      80      90     100
YNTLNPEASASPCCVPQDLEPLTILYYVGRTPKVEQLSNMVVKSKCS.
```

20. The protein of claim 16 comprising the amino acid sequence:

```
      10      20      30      40      50
LYVEFKDVGWQNWVIAPOGYMANCYGECPPYPLTEILNGSNHAIL
      60      70      80      90     100
QTLVHSIEPEDIPLPCCVPTKMSPVAMLYLNDQSTVVLKQNYQEMTVVGCGR.
```

21. The protein of claim 16 comprising the amino acid sequence:

```
      10      20      30      40      50
LYVDFSDVGWDDWIVAPLGYDAYYCHGKCPFLADHFNSTNHAVVQ
      60      70      80      90     100
TLVNNNNPGKVPKACCVPTQLDSISMLFYDNNNDNVVLRHYENMAVDECGCR.
```

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22. The protein of claim 16 comprising the amino acid sequence:

10	20	30	40	50
LYIDFKRDLGWKVVHEPKGYAANFCAGACPYLWSADTQHSRVLALYN				
60	70	80	90	100
TANPEASAAPCCVPQDLEPLTILYYVGRTPKVEQLSNMVVKSKCS.				

23. A DNA sequence encoding the amino acid sequence of one of the chains of the construct of claim 1 or 3.

24. A prokaryotic cell engineered to express the DNA sequence encoding the amino acid sequence of one of the chains of the construct of claim 1 or 3.

25. The prokaryotic cell of claim 24 wherein said cell is E. coli.

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26. A method of producing a protein construct having the activity of transforming growth factor-beta, said method comprising the steps of:

- (a) transforming a prokaryotic host cell with a vector comprising the DNA sequence of claim 23;
- (b) culturing said transformed host cell to express said protein construct;
- (c) purifying said protein construct; and
- (d) activating said protein construct by oxidation in vitro to produce a two chain disulfide-linked transforming growth factor-beta analog, said activated protein construct having an anti-proliferative effect on mammalian epithelial cells in vitro.

27. The method of claim 26 wherein said activated protein comprises a dimer.

28. The method of claim 26 wherein said activated protein comprises fewer than 9 cysteine residues.

29. The method of claim 28 wherein said activated protein comprises 6-8 cysteine residues.

30. The method of claim 28 wherein said activated protein comprises 8 cysteine residues.

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31. The method of claim 28 wherein said activated protein comprises 6 cysteine residues.

32. The method of claim 28 wherein said activated protein is unglycosylated.

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FIGURE 1

	-14	1	10	20
TGF-B1	ALDTNYCFSSTEKNCCVRQLYIDFRKDLGWK-WIHEPKGYH			
	*** **	*** * *	*** * *	*** * *
TGF-B2	ALDAAYCFRNVODNCCLRPLYIDFKRDLGWK-WIHEPKGYN			
	30	40	50	60
TGF-B1	ANFCLGPCPYIWS--L-DTQ--Y-SKVLALYNQHNPAS-A			
	*** * *	*** * *	*** * *	*** * *
TGF-B2	ANFCAGACPYLWS--S-DTQ--H-SRVLSLYNTINPEAS-A			
	70	80	90	100
TGF-B1	APCCVPQALEPLPIVYYVGRKPKVEQL-SNMIVRSCKCS			
	*** * *	*** * *	*** * *	*** * *
TGF-B2	SPCCVSQDLEPLTILYYIGKTPKIEQL-SNMIVKSKCS			

	-14	1	10	20
TGF-B1	ALDTNYCFSSTEKNCCVRQLYIDFRKDLGWK-WIHEPKGYH			
	*****	* * * * *	*****	* * * * *
TGF-B3	ALDTNYCFRNLEENCCVRPLYIDFRQDLGWK-WVHEPKGY			
	30	40	50	60
TGF-B1	ANFCLGPCPYIWS--L-DTQ--Y-SKVLALYNQHNPAS-A			
	*****	* * *	* * * * *	* * * * *
TGF-B3	ANFCSGPCPYLRS--A-DTT--H-STVLGLYNTLNPEAS-A			
	70	80	90	100
TGF-B1	APCCVPQALEPLPIVYYVGRKPKVEQL-SNMIVRSCKCS			
	*****	* * * * *	*****	* * * * *
TGF-B3	SPCCVPQDLEPLTILYYVGRTPKVEQL-SNMVVKSKCS			

	-14	1	10	20
TGF-B1	ALDTNYCFSSTEKNCCVRQLYIDFRKDLGWK-WIHEPKGYH			
		* * * * *	* * *	* * *
Vg1	KRSYSKLPFTASNICKRHLVVEFK-DVGWQNWVIAPOGYM			
	30	40	50	60
TGF-B1	ANFCLGPCPYIWS--L-DTQ--Y-SKVLALYNQHNPAS-A			
	* * * * *	*	*	*
Vg1	ANYCYGECPYPLTEILNGSN--H-AILQTLVHSIEPED-IP			
	70	80	90	100
TGF-B1	APCCVPQALEPLPIVYYVGRKPKVEQL-SNMIVRSCKCS			
	*****	* *	* *	* * *
Vg1	LPCCVPTKMSPISMLFYDNNDNVVLRHYENMAVDECGCR			

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FIGURE 1 (cont'd.)

	-14	1	10	20
TGF-B1	ALDTNYCFSSTEKNCCVRQLYIDFRKDLGWK-WIHEPKGYH			
		*	** **	** ** *
DPP	HARRPTRRKNHDDTCRRHSLYVDFS-DVGWDDWIVAPLGVD			
	30	40	50	60
TGF-B1	ANFCLGPCPYIWS--L-DTQ--Y-SKVLALYNQHNPAS-A			
	* * * *	*	* * *	***
DPP	AYYCHGKCPFPLADHFNSTN--H-AVVQTLVNNNNPGK-VP			
	70	80	90	100
TGF-B1	APCCVPQALEPLPIVYVGRKPKVEQL-SNMIVRSCKCS			
	***** *	*	*	* * *
DPP	KACCVPTQLDSVAMLYLNDQSTVVLKNYQEMTVVGCGCR			

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FIGURE 2A

```

      10      20      30      40      50
ATGAAAGCAATTTTCGTACTGAAAGGTTCACTGGACAGAGATCTGGACTC
M K A I F V L K G S L D R D L D S
---MLE leader--->                               BglII

      60      70      80      90     100
TCGTCTGGATCTGGACGTTTCGTACCGACCACAAAGACCTGTCTGATCACC
R L D L D V R T D H K D L S D H

      110     120     130     140     150
TGGTTCTGGTCGACCTGGCTCGTAACGACCTGGCTCGTATCGTTACTCCCG
L V L V D L A R N D L A R I V T P
      Sall                                         SmaI

      160     170     180     190     200
GGTCTCGTTACGTTGCGGATCTGGAATTCATGGCTGACAACAAATTCAA
G S R Y V A D L E F M A D N K F N
                        EcoRI  ----Fb-Fb leader--

      210     220     230     240     250
CAAGGAACAGCAGAACGCGTTCTACGAGATCTTGCACCTGCCGAACCTGA
K E Q Q N A F Y E I L H L P N L
->          MluI          BglII          BspMI

      260     270     280     290     300
ACGAAGAGCAGAAGGACGGCTTCATCCAGAGCTTGAAGGATGAGCCCTCT
N E E Q K D G F I Q S L K D E P S

      310     320     330     340     350
CAGTCTGCGAATCTGCTAGCGGATGCCAAGAACTGAACGATGCGCAGGC
Q S A N L L A D A K K L N D A Q A
          NheI                               FspI

      360     370     380     390     400
ACCGAAATCGGATCAGGGGCAATTCATGGCTGACAACAAATTCAACAAGG
P K S D Q G Q F M A D N K F N K

      410     420     430     440     450
AACAGCAGAACGCGTTCTACGAGATCTTGCACCTGCCGAACCTGAACGAA
E Q Q N A F Y E I L H L P N L N E
      MluI          BglII          BspMI+

```

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FIGURE 2A (cont'd.)

460 470 480 490 500
 GAGCAGAAGGACGGCTTCATCCAGAGCTTGAAGGATGAGCCCTCTCAGTC
 E Q K D G F I Q S L K D E P S Q S

510 520 530 540 550
 TCGGAATCTGCTAGCGGATGCCAAGAACTGAACGATGCGCAGGCACCGA
 A N L L A D A K K L N D A Q A P
 NheI FspI <-leader

560 570 580 590 600
 AGGATCCTAATGGGCTGTACATCGATTTCCGTAAAGACCTGGGTTGGAAG
 K D P N G L Y I D F R K D L G W K
 -- ===hinge=== --TGF-beta with 6 cys----->
 BamHI

610 620 630 640 650
 TGGATTCATGAACCTAAGGGTTACCATGCCAACTTCTGCCTGGGCCCTTG
 W I H E P K G Y H A N F C L G P C
 MstII BstEII ApaI EcoO

660 670 680 690 700
 TCCGTACATCTGGTCTCTGGATACCCAGTACTCCAAGGTGCTGGCTCTGT
 P Y I W S L D T Q Y S K V L A L
 ScaI BstXI

710 720 730 740 750
 ACAATCAGCATAACCCGGGGGCTAGCGCAGCTCCGTGCTGTGTTCCACAGGC
 Y N Q H N P G A S A A P C C V P Q A
 SmaI NheI StuI

760 770 780 790 800
 CTTGGAACCGCTGCCGATCGTCTATTACGTCGGCCGTAAGCCTAAGGT
 L E P L P I V Y Y V G R K P K V
 PvuI EagI MstII

810 820 830 840 850
 TGAACAGCTGTCTAACGTGATTGTGCGCAGTTGCAAGTGCTCTTAAGGATCC
 E Q L S N V I V R S C K C S *
 PvuII FspI AflIIBamHI

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FIGURE 2B

```

      10      20      30      40      50
ATGAAAGCAATTTTCGTA CTGAAAGGTTCACTGGACAGAGATCTGGACTC
M K A I F V L K G S L D R D L D S
|---MLE leader--->                               BglII

      60      70      80      90      100
TCGTCTGGATCTGGACGTTCTGTACCGACCACAAAGACCTGTCTGATCACC
R L D L D V R T D H K D L S D H

      110     120     130     140     150
TGGTTCTGGTCGACCTGGCTCGTAACGACCTGGCTCGTATCGTTACTCCC
G
L V L V D L A R N D L A R I V T P
      Sall
SmaI

      160     170     180     190     200
GGTCTCGTTACGTTGCGGATCTGGAATTCATGGCTGACAACAAATTCAA
G S R Y V A D L E F M A D N K F N
      EcoRI |----Fb-Fb leader--

      210     220     230     240
250
CAAGGAACAGCAGAACGCGTTCTACGAGATCTTGACCTGCCGAACCTGA
K E Q Q N A F Y E I L H L P N L
->                               MluI           BglII           BspMI

      260     270     280     290     300
ACGAAGAGCAGAAGGACGGCTTCATCCAGAGCTTGAAGGATGAGCCCTCT
N E E Q K D G F I Q S L K D E P S

      310     320     330     340     350
CAGTCTGCGAATCTGCTAGCGGATGCCAAGAACTGAACGATGCGCAGGC
Q S A N L L A D A K K L N D A Q A
      NheI                               FspI

      360     370     380     390     400
ACCGAAATCGGATCAGGGGCAATTCATGGCTGACAACAAATTCAACAAGG
P K S D Q G Q F M A D N K F N K

```

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FIGURE 2B (cont'd.)

410 420 430 440 450
 AACAGCAGAACGCGTTCTACGAGATCTTGCACCTGCCGAACCTGAACGAA
 E Q Q N A F Y E I L H L P N L N E
 MluI BglII BspMI+

460 470 480 490 500
 GAGCAGAAGGACGGCTTCATCCAGAGCTTGAAGGATGAGCCCTCTCAGTC
 E Q K D G F I Q S L K D E P S Q S

510 520 530 540 550
 TGCGAATCTGCTAGCGGATGCCAAGAACTGAACGATGCGCAGGCACCGA
 A N L L A D A K K L N D A Q A P
 NheI FspI <-leader

560 570 580 590 600
 AGGATCCTAATGGGTGCTGCGTGCGTCAGCTGTACATCGATTTCCGTAAA
 K D P N G C C V R Q L Y I D F R K
 --|===hinge===|--TGF-beta with 8 cys-----
 BamHI

660 670 680 690 700
 GACCTGGGTTGGAAGTCCGTACATCTGGTCTCTGGATACCCAGTACTCCAAG
 D L G W K P Y I W S L D T Q Y S K
 ScaI BstXI

710 720 730 740 750
 GTGCTGGCTCTGTACAATCAGCATAACCCGGGGGCTAGCGCAGCTCCG
 V L A L Y N Q H N P G A S A A P
 SmaI NheI

760 770 780 790 800
 TGCTGTGTTCCACAGGCCTTGGAAACCGCTGCCGATCGTCTATTACGTCGGC
 C C V P Q A L E P L P I V Y Y V G
 StuI PvuI EagI

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FIGURE 2B (cont'd.)

810	820	830	840	850
CGTAAGCCTAAGGTTGAACAGCTGTCTAACGTGATTGTGCGCAGTTGCA				
R K P K V E Q L S N V I V R S C				
MstII	PvuII		FspI	

860
AGTGCTCTTAAGGATCC
K C S *
AflIII BsmHI

INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US90/06006**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): A61K 37/24, 37/36; C07K 3/00, 13/00, 15/00, 17/00 U.S. CL.: 530/399, 324, 350, 351		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
U.S. Class	530/399, 324, 350, 351	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	Nature, Volume 316, issued 22 August 1985 DERYNCK ET AL. , "Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells," pages 701-705, see entire document.	1-6, 8, 16, 17
Y	US, A, 4,572,798 (KOTHS ET AL.) 25 February 1986, see entire document.	1-6, 8, 16, 17
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>[*] Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
31 January 1991		04 MAR 1991
International Searching Authority		Signature of Authorized Officer
ISA/US		 Shelly J. Guest (vsh)

Form PCT/ISA/210 (second sheet) (Rev. 11-87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET**V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹**

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this international application as follows:

See attachment to PCT Telephone Memorandum.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers: **1-22 Telephone practice.**
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.

